Ineffective erythropoiesis caused by binucleated late-stage erythroblasts in mDia2 hematopoietic specific knockout mice

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Supplemental Methods

Materials

Iscove modified Dulbecco medium (IMDM, 12440-046) was purchased from Gibco. Fetal bovine serum (Cat#06200) and bovine serum albumin (Cat#09300) were purchased from Stem Cell Technologies, Recombinant human insulin (I9278) and recombinant human holo-transferrin (T1283) were ordered from Sigma-Aldrich. Penicillin-streptomycin and L-glutamine were purchased from HyClone. Erythropoietin (Epo, NDC 59676-310-00) was purchased from Amgen. The biotin mouse lineage panel (Cat#559971) was purchased from BD Pharmingen to mark lineage positive cells or to positively or negatively select lineage positive cells from mouse bone marrow cells. The phycoerythrin (PE)–conjugated antibodies rat anti-mouse CD11b/Mac1 (M1/70, Cat#553311), PE-CD8α (53-6.7, Cat#553033), PE-CD45.1 (A20, Cat#553776), Fluorescein isothiocyanate (FITC)-CD45.1 (A20, Cat#11-0453-85) and Cy5-Annexin V (Cat#559934) were obtained from BD Pharmingen. Allophycocyanin (APC)-conjugated anti-mouse CD45.2 (109814) were purchased from Biolegend. FITC-conjugated CD71 (R17217, SC-52504) was purchased from Santa Cruz Biotech. PE-human/mouse CD44 (IM7, 12-0441-82), PE-mouse Ter119 (Ter119, 12-5921-83), APC-mouse Ter119 (Ter119, 17-5921-83), PE-Cyanine 7(PE-Cy7)-mouse CD4 (GK1.5, 25-0041-81), PE-Cy7-Gr1 (RB6-8C5, 25-5931-81), Pacific Blue-human/mouse B220 (RA3-6B2, 11-0452-82) and FITC-CD45.2 (104, Cat#11-0454-85) antibodies were purchased from eBiosciences. Hoechst 33342, 4,6 diamidino-2-phenylindole, and rhodamine phalloidin were purchased from Molecular Probes, Invitrogen. Polyclonal anti-mouse mDia2 antibody was generated by immunizing rabbits with mDia2 proteins according to standard procedure.

RNA extraction and quantitative RT-PCR

The total RNA was extracted using Trizol reagent according to the manufacturer's protocol (life technologies). 1μg total RNA was used for the reverse transcription by qScript cDNA supermix (Quanta Biosciences). Relative mRNA expression levels of various genes were assessed by QRT-PCR. Each template was tested in triplicate. The abundance of each gene was normalized to 18s rRNA. The primer sequences used in this study were *mDia1* forward 5'-GGACTGCTTCTGGACAAAGG-3'; reverse 5'-TCTCCACCTTCTTGATCCTTCT-3'; *mDia3* forward 5'- AATCTTCTGGAAGCCCTACAGT; reverse 5'-GGCCGTCTGTTATCTGGATTTC-3'; *mDia2* forward 5'- AGCCTTGACTTCAGCTGGAG-3'; reverse 5'- GGTGAAGCCTGAAGTCCAAA-3'; 18s rRNA forward 5'-GCAATTATTCCCCATGAACG-3'; reverse 5'- GGCCTCACTAAACCATCCAA-3'.

Flow cytometric and cell cycle analysis

For bone marrow, mouse bone marrow cells were flushed using a syringe with 30.5G needle and passed through a 40μm cell strainer. The single cell suspensions were labeled with appropriate antibodies in FACS buffer (1×PBS containing 0.5% BSA and 2mM EDTA) for 15 min at room temperature, washed and resuspended in FACS buffer. Propidium iodide (PI) or 4', 6 diamidino-2-phenylindole (DAPI) was added at the final step to exclude the dead cells. The cells were then analyzed by BD FACSCanto II flow analyzer and the data were further analyzed by FlowJo software.

For peripheral blood, approximately 50~80μl tail vein or retro-orbital blood from mice were collected in BD microtainer tubes with EDTA supplied by BD Biosciences. The blood was first assayed by complete blood count (CBC) test, and was then resuspended in red blood cell (RBC)

lysis buffer for 5-7 minutes on ice with intermittent mixing. Immediately after incubation, the RBC lysed cells were washed with ice cold PBS and passed through a 40μm cell strainer, which were then labeled with appropriate antibodies for flow cytometry analysis as detailed above.

For spleen, after measuring the weight, the whole spleen was dispersed into single cell suspensions of splenocytes by homogenization using the frosted ends of the slides and passing through 40μm cell strainer. The cells were then labeled with appropriate antibodies for further flow cytometric analysis.

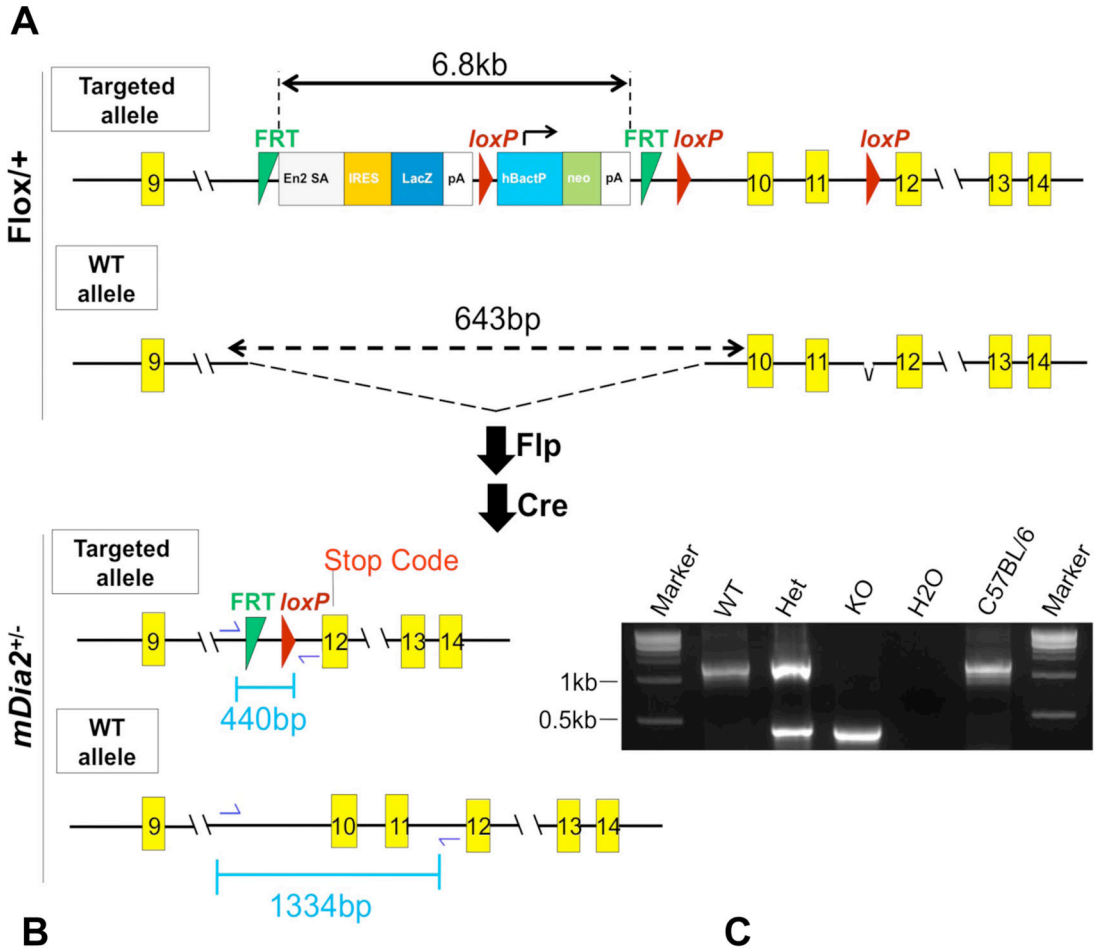
To analyze the cell cycle distribution of erythroblast cells from bone marrow and spleen, the single cell suspensions were incubated with phycoerythrin (PE) or allophycocyanin (APC)–conjugated Ter119 antibodies, washed with FACS buffer and resuspended in IMDM containing Vybrant DyeCycle Violet (V35003) from Molecular Probes, Invitrogen. The cells were kept at 37˚C for 30 min. Propidium iodide (PI) was added prior to flow cytometry acquisition to exclude the dead cells. The DNA low population defined as RBCs was eliminated from Ter119 positive cells and the DNA content distribution of remaining nucleated cells (defined as erythroblasts) are then analyzed by histogram and Watson (Pragmatic) model with FlowJo software.

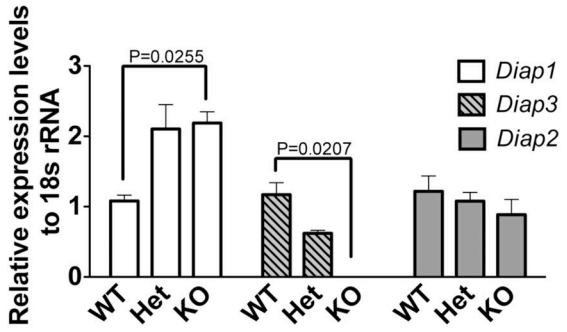
Mice genotyping

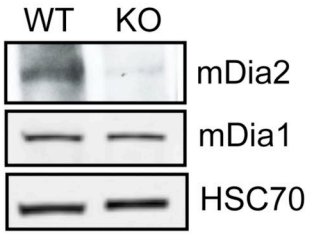
The genotype of *Diap3* floxed alleles was assayed by genomic PCR using the follow primers: forward: 5'- CTACCAACCTACCCATCCATC-3'; reverse: 5'- CGAGAGCATTTATGAGCTGCATACAA-3'. Primers for mDia2-deficient allele genotyping, forward: 5'- TTGGCTGTTCTGGAAGTTGC-3'; reverse: 5'- CAGCAGCATTCCTTTCCACA-

3’. For Flp transgenic mice genotyping, the primers are forward: 5’-CACCACCTAAGGTGCTTGTTC-3’; reverse: 5’-CTGCTTCTTCCGATGATTCG-3’ (PCR product ~370bp). The Cre primers for both Mx1-Cre and E2A-Cre are forward: 5’-CGTACACCAAAATTTGCCTGC-3’; reverse: 5’-CTAGAGCCTGTTTTGCACGTT-3’ (PCR product ~390bp). Primers for Vav-Cre are forward: 5’-AGATGCCAGGACATCAGGAACCTG-3’; reverse: 5’-ATCAGCCACACCAGACACAGAGATC-3’ (PCR product ~240bp). All the experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees at Northwestern University.

Supplemental Figures







D

	WT	Het	KO
Female	9	18	0
Male	9	16	0

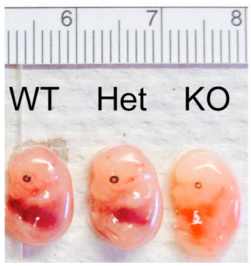


Figure S1. Generation of conditional mDia2 knockout mice and the phenotypes of mDia2$^{fl/fl}$ E2A-Cre mice.

(**A**) Schematic representations of the wide type and targeted mDia2 alleles. Positions of primers for genomic DNA PCR are indicated by blue arrows and the expected length of the PCR band are shown in aqua lines on the lower panel. The genotyping PCR on a 2% agarose gel was shown. (**B**) Quantification of mRNA levels of *Diap1, 2* and *3* of indicated E9 live embryos by a quantitative real time PCR assay. WT (mDia2$^{+/+}$ E2A-Cre): N=2, Het (mDia2$^{fl/+}$ E2A-Cre): N=4, KO (mDia2$^{fl/fl}$ E2A-Cre): N=2. (**C**) Western blotting analysis of mDia2 and mDia1 from E9 embryos lysates of the wild type (WT) and mDia2$^{fl/fl}$ E2A-Cre (KO). HSC70 was used as a loading control. (**D**) Genotype analysis of the offspring from mDia2$^{fl/+}$ E2A-Cre heterozygous mating. **(E**) Representative photographs show the conditional knockout of mDia2 in the early stage of embryonic development causing embryonic lethality at approximately E12.5.

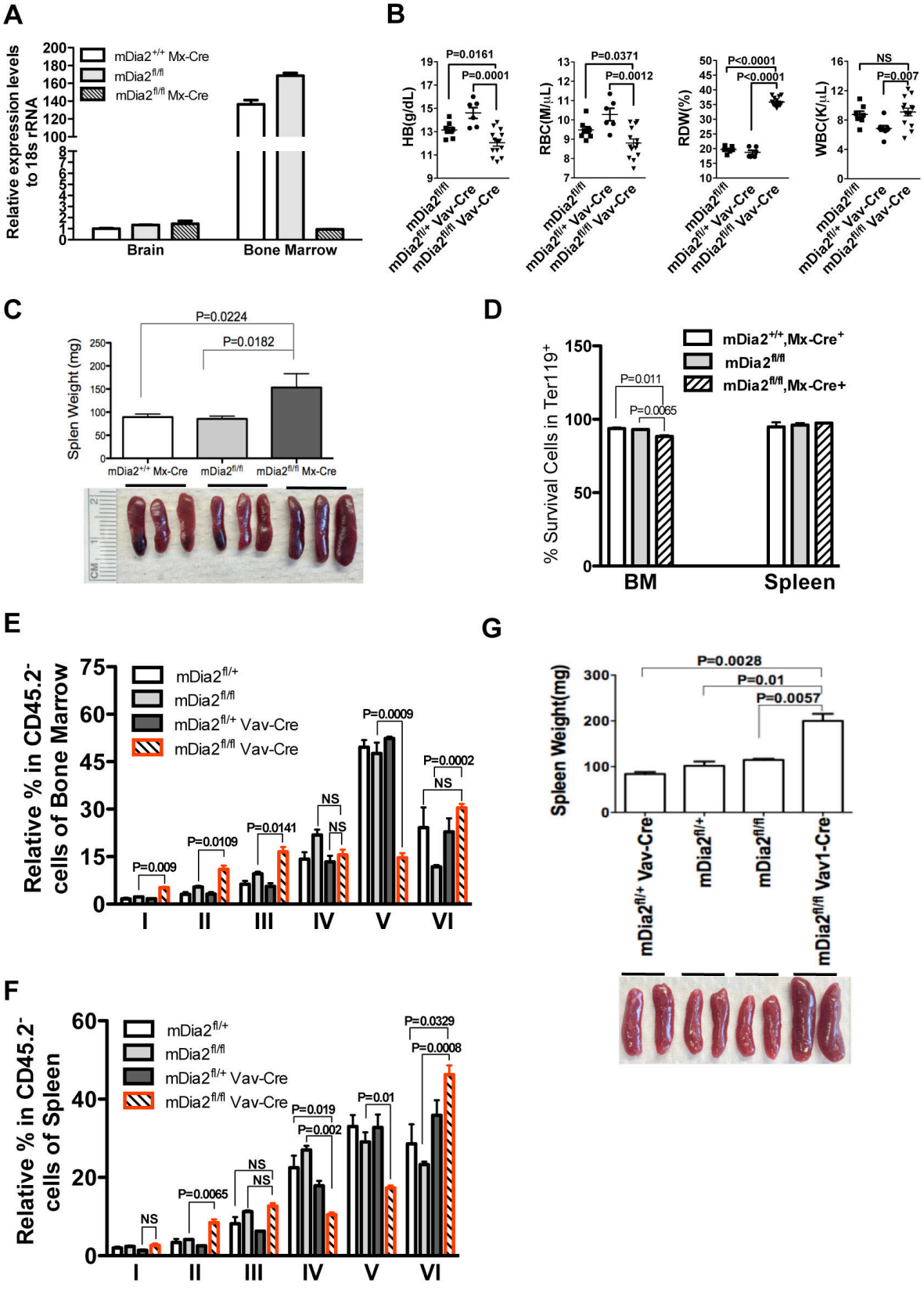

A
Relative expression levels to 18s rRNA
mDia2+/+ Mx-Cre
mDia2fl/fl
mDia2fl/fl Mx-Cre
Brain
Bone Marrow
B
HB(g/dL)
RBC(M/μL)
RDW(%)
WBC(K/μL)
P=0.0161
P=0.0001
P=0.0371
P=0.0012
P<0.0001
P<0.0001
NS
P=0.007
mDia2fl/fl
mDia2fl/+ Vav-Cre
mDia2fl/fl Vav-Cre
C
Splen Weight (mg)
P=0.0224
P=0.0182
mDia2+/+ Mx-Cre
mDia2fl/fl
mDia2fl/fl Mx-Cre
D
% Survival Cells in Ter119+
mDia2+/+,Mx-Cre+
mDia2fl/fl
mDia2fl/fl,Mx-Cre+
P=0.011
P=0.0065
BM
Spleen
E
Relative % in CD45.2- cells of Bone Marrow
mDia2fl/+
mDia2fl/fl
mDia2fl/+ Vav-Cre
mDia2fl/fl Vav-Cre
P=0.009
P=0.0109
P=0.0141
NS
NS
P=0.0009
P=0.0002
NS
I
II
III
IV
V
VI
F
Relative % in CD45.2- cells of Spleen
mDia2fl/+
mDia2fl/fl
mDia2fl/+ Vav-Cre
mDia2fl/fl Vav-Cre
NS
P=0.0065
NS
NS
P=0.019
P=0.002
P=0.01
P=0.0329
P=0.0008
I
II
III
IV
V
VI
G
Spleen Weight(mg)
P=0.0028
P=0.01
P=0.0057
mDia2fl/+ Vav-Cre
mDia2fl/+
mDia2fl/fl
mDia2fl/fl Vav1-Cre

Figure S2. mDia2 hematopoietic specific knockout mice develop ineffective erythropoiesis and splenomegaly.

(A) Relative mDia2 mRNA levels in cells from brain and bone marrow of age matched control ($mDia2^{fl/fl}$ and $mDia2^{+/+}$Mx-Cre) and hematopoietic specific knockout ($mDia2^{fl/fl}$Mx-Cre) mice 10 weeks after the first poly-IC injection. (B) Peripheral blood hemoglobin, RBC, RDW and WBC count from 6-8 weeks aged control ($mDia2^{fl/fl}$, N=6), hematopoietic specific heterozygous ($mDia2^{fl/+}$Vav-Cre, N=6), and knockout ($mDia2^{fl/fl}$Vav-Cre, N=13) mice. (**C**) Quantification of spleen weight and representative photographs from mDia2 control and Mx-Cre conditional knockout mice. (**D**) Statistical summary of flow cytometric analysis of the relative percentage of the survival cells in Ter119 positive cells derived from bone marrow and spleen of the indicated mice. (**E-G**) Quantifications of I-VI populations, assayed by flow cytometric analysis of CD44 expression and forward scatter as in Figure 1C and 1E, were shown in (E) for bone marrow and in (F) for spleen from $mDia2^{fl/fl}$Vav-Cre and control mice. Quantitative analysis of spleen weight from these mice was shown in (G). All the experiments were repeated at least three times.

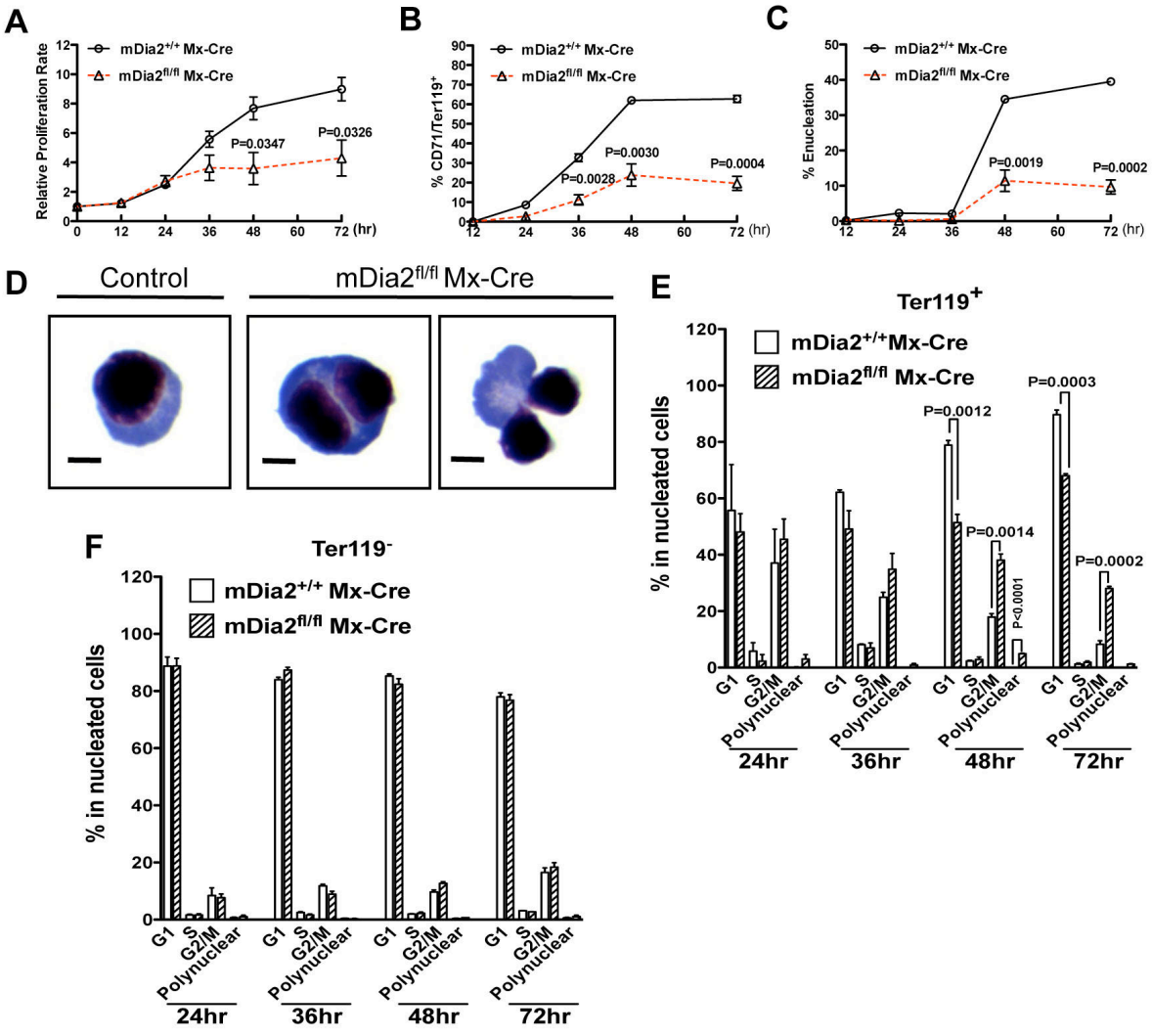

A
Relative Proliferation Rate
mDia2+/+ Mx-Cre
mDia2fl/fl Mx-Cre
P=0.0347
P=0.0326
(hr)
B
% CD71/Ter119+
P=0.0030
P=0.0028
P=0.0004
C
% Enucleation
P=0.0019
P=0.0002
D
Control
mDia2fl/fl Mx-Cre
E
Ter119+
% in nucleated cells
P=0.0012
P=0.0014
P=0.0003
P=0.0002
P<0.0001
G1
S
G2/M
Polynuclear
24hr
36hr
48hr
72hr
F
Ter119-

Figure S3. Impaired terminal erythropoiesis and formation of binucleated erythroblasts of mDia2 deficient hematopoietic progenitor cells in vitro

(**A-C**) Lineage negative bone marrow progenitor cells were purified from the indicated mice and cultured in erythropoietin containing medium. Relative cell proliferation rate during in vitro erythroid differentiation culture was shown in (A) at indicated time point. The percentages of CD71 and Ter119 double positive cells were shown in (B) for differentiation assay. The percentages of enucleation were presented in (C), respectively. (**D**) Wright-Giemsa stains of representative cultured late stage erythroblasts of (A-C) from mDia2$^{+/+}$Mx-Cre (control) and mDia2$^{fl/fl}$Mx-Cre mice at 48hr. Scale bars: 3 μm. (**E-F**) Cell cycle analysis of the Ter119 positive (E) and negative cells (F) from (A) at indicated time point.

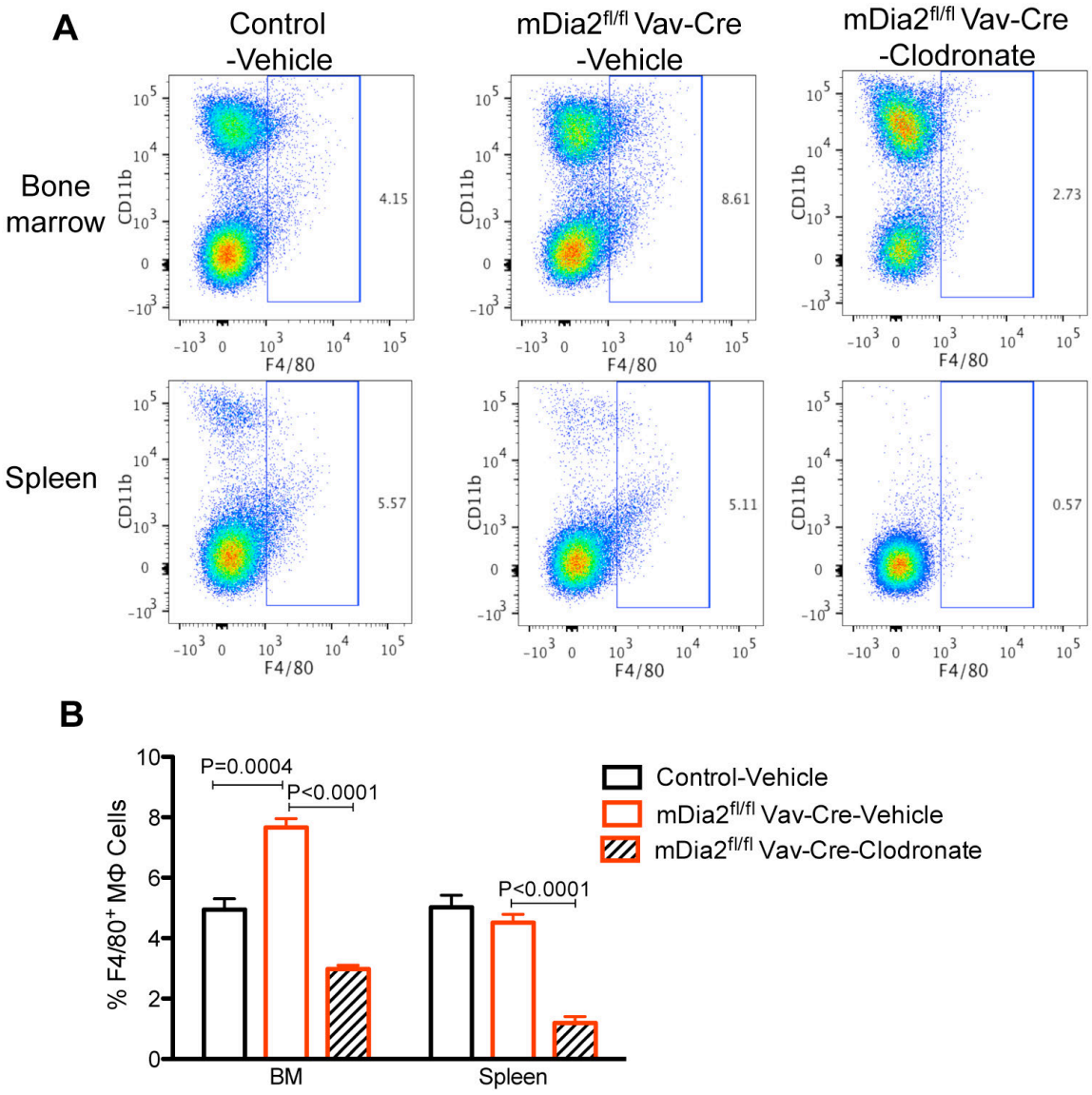


Figure S4 Clodronate eliminates macrophage in bone marrow and spleen.

Flow cytometric analysis of bone marrow (BM) and spleen cells after clodronate treatment by staining cells with CD11b and F4/80. Representative plots are presented in (A). Quantitative analysis of the percentages of macrophage is shown in (B). N=5 in each group.